# Monitoring cellular movement *in vivo* with photoconvertible fluorescence protein "Kaede" transgenic mice

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**Kaede is a photoconvertible fluorescence protein that changes from green to red upon exposure to violet light. The photoconversion of intracellular Kaede has no effect on cellular function. Using transgenic mice expressing the Kaede protein, we demonstrated that movement of cells with the photoconverted Kaede protein could be monitored from lymphoid organs to other tissues as well as from skin to the draining lymph node. Analysis of the kinetics of cellular movement revealed that each subset of cells in the lymph node, such as CD4$^+$ T, CD8$^+$ T, B, and dendritic cells, has a distinct migration pattern *in vivo*. Thus, the Kaede transgenic mouse system would be an ideal tool to monitor precise cellular movement *in vivo* at different stages of immune response to pathogens as well as in autoimmune diseases.**

cell migration | dendritic cells | lymphocyte | photoconvertible protein

Lymphocytes and other cells of the immune system are mobile and move around the whole body for surveillance (1). Effective host defense against pathogens requires both innate and adaptive arms of the immune response (2, 3). The transition from the innate to the adaptive arm of the immune response is a critical step for the clearance of pathogens and is initiated by the interaction between antigen-presenting cells and a very small number of antigen-specific T and B cells in lymphoid organs (2, 3). For the successful interaction to establish effective immune response, cooperative migration of antigen-presenting cells from inflammatory sites to the lymph node and recruitment of a sufficient number of rare antigen-specific lymphocytes to the same lymph node are required (4, 5). Thus, the regulation of cellular movement in the body is vital to fighting infections.

The development of the two-photon laser intravital microscope has enabled us to observe cellular movement *in vivo* (6, 7). It has also enabled us to monitor interactions between dendritic cells and T cells, T cells and B cells, and other types of cells in various lymphoid organs. This instrument, when used with new methods to visualize different cells (8), is an important tool for the analysis of immune responses *in vivo*. However, the two-photon laser intravital microscope is applicable to only a limited area of the organ of interest.

Classical studies involving injection of *ex vivo* labeled cells into animals have demonstrated the continuous recirculation of lymphocytes between the bloodstream and the lymph system (9, 10) and the dynamic changes in migration and emigration of cells in lymphoid organs as well as in the circulation at various stages of immune response (11–13). However, this conventional cell transfer system has limitations. Because of the low frequency of transferred cells in host, it is difficult to use this system for small populations, and this system cannot be applied to all cell types because mechanical stress and/or incubation during cell isolation and labeling processes *ex vivo* stimulate some type of cells. To overcome these problems, *in situ* cell labeling has been used for several organs and tissues, such as the thymus (14), mesenteric lymph node, spleen (15), and leukocytes (16, 17). However, the direct injection of fluorescence substrate into organs inevitably caused inflammatory reactions in the tissue. Thus, these methods have limited applications, and current technologies do not allow us to observe natural cellular movement in the body. In this kind of situation, various genetically altered mouse lines and mAbs specific to cell-surface molecules were established, and new insights into cellular migration of the immune system in the steady state and during immune response have been provided (4, 5, 18, 19). However, almost all of those studies were done by combination with a conventional cell transfer system, and information was limited to the general homing pattern of cells with no information on cellular emigration from tissue. Therefore, establishment of a novel system to track immune cells in mice will be an ideal tool for cell migration analysis, will contribute to visualization of cellular movement in the whole body, and will clarify its molecular mechanisms.

Here we report a system to monitor cellular movement *in vivo* with minimum manipulation of the transgenic mouse expressing Kaede, a new photoconvertible fluorescence protein (20). Cells expressing Kaede can be marked by exposure to light without exerting any effects on cellular function. It is also possible to expose the lymph node and other organs to light with minimum manipulation. This system allows us to analyze cellular movement *in vivo*, including both immigration and emigration of cells in target organs. In an initial analysis of normal mouse, we demonstrated that cells are moving rapidly within lymphoid organs and some cells are migrating from nonlymphoid organs to lymphoid organs. Moreover, the migration of cells *in vivo* is regulated in a cell-type-specific manner in that each cell type, CD4$^+$ T, CD8$^+$ T, B, and dendritic cells, has a distinct replacement rate in the lymph node. Thus, we believe that Kaede transgenic mice can overcome many of the problems besetting previous technologies and can be used to monitor cellular movement *in vivo* during the generation and effector phase of protective immune response as well as the onset of the autoimmune phenomenon.

## Results

**Photoconversion of Kaede in Cells and in Transgenic Mouse.** Kaede is a photoconvertible GFP cloned from stony coral (20). Exposure

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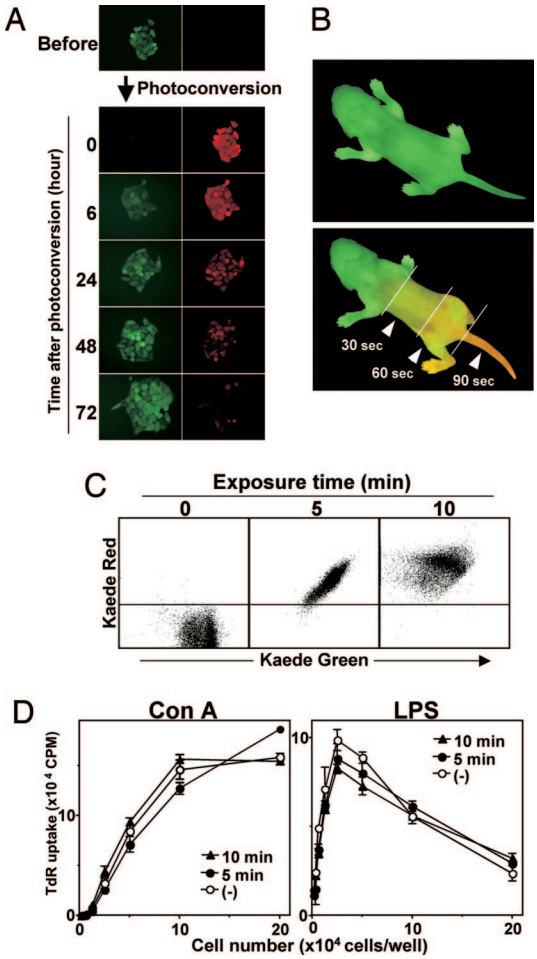


**Fig. 1.** Photoconversion of Kaede in cells and in transgenic mice. (*A*) Kaede-expressing HeLa cells were photoconverted with UV light as described in *Materials and Methods*. The photoconverted Kaede signal was monitored for the indicated times after photoconversion. (Scale bar: 30 $\mu$m.) (*B*) A neonatal Kaede transgenic mouse (*Upper*) was exposed to violet light as described in *Materials and Methods* for indicated times (*Lower*) and observed with a fluorescence stereoscopic microscope. (*C*) Spleen cells from Kaede transgenic mice were exposed to violet light for 0, 5, or 10 min and subjected to flow cytometry. (*D*) The indicated numbers of spleen cells were stimulated with either Con A (5 $\mu$g/ml) or LPS (10 $\mu$g/ml), and cell proliferation was measured by [$^3$H]thymidine incorporation as described in *Materials and Methods*. Mean ± SD of triplicate wells is shown. Results are representative of two experiments.

of Kaede to light induces unique peptide cleavage and subsequent formation of a double bond within the Kaede chromophore and changes its excitation and emission wavelengths (21). This change from green to red fluorescence can be detected easily with a conventional fluorescence microscope (Fig. 1*A*). Cell proliferation diluted photoconverted Kaede with newly synthesized nonphotoconverted Kaede, and after several cell divisions the detection of red fluorescence became difficult [Fig. 1*A* and supporting information (SI) Fig. S1]. However, it should be noted that the exposure of Kaede to light permanently changed its structure, and photoconverted Kaede had a very long biological half-life in lymphocytes *in vivo* (Fig. S2) as well as in neuronal cells, as shown in a previous report (20). Transgenic mice carrying Kaede cDNA under the CAG promoter were established (our unpublished observations). These mice expressed photoconvertible Kaede in all of the cell types analyzed (our unpublished observations and Fig. 1*B*) with no abnormality in their growth and reproduction. More importantly, expression of the Kaede protein and the photoconverting procedure had no effect on the homing capacity of both T and B cells to lymphoid organs (Fig. S3).

Spleen cells from Kaede transgenic mice were exposed to violet light for 0, 5, and 10 min, and the cells were analyzed for the presence of red fluorescence and their capacity to proliferate *in vitro*. As shown in Fig. 1*C*, all cells had both red and green signals after exposure for 5 min because of the presence of both photoconverted and nonphotoconverted Kaede in the cells. After exposure for 10 min, some cells started to lose green fluorescence because of the loss of nonphotoconverted Kaede in the cells. Exposure of the cells to violet light for 5 or 10 min had no effect on their capacity to proliferate upon stimulation with Con A (T cells) or LPS (B cells) (Fig. 1*D*). Thus, we used a 5-min exposure of cells to violet light to convert Kaede *in vivo* throughout this study.

**Migration of Lymphocytes *in Vivo* in the Steady State.** To monitor cellular migration *in vivo*, the inguinal lymph node was visualized by making a small incision on the skin and exposing it to violet light for 5 min. This procedure reproducibly converted 100% of the inguinal lymph node cells to express red fluorescence (Fig. 2*A*). In some experimental mice, the wound was surgically closed after photoconversion and lymph node cells were analyzed on days 1, 3, and 7. As shown in Fig. 2*B*, on day 1 after photoconversion, 76% of cells in the photoconverted inguinal lymph node had no photoconverted Kaede. Cells with photoconverted Kaede were found, albeit at a low percentage, in all lymphoid organs and peripheral blood. These results indicate that lymph node cells were rapidly replaced and migrated out to the whole body. It should be noted that, upon photoconversion of the inguinal lymph node, a large number of photoconverted cells were found in the ipsilateral axillary lymph node.

When mice were treated with the sphingosine-1 phosphate receptor agonist FTY-720 (22, 23) after photoconversion of the inguinal lymph node, the appearance of photoconverted Kaede-negative cells in the inguinal lymph node and the number of photoconverted Kaede-positive cells in other lymph nodes were drastically reduced (Fig. 2*C*). These results indicate that movement of cells with photoconverted Kaede-positive cells *in vivo* reflects physiological immigration and emigration of cells in lymphoid organs.

Analysis of mice on days 3 and 7 after photoconversion of the inguinal lymph node revealed that only low percentages of photoconverted Kaede-positive cells were found in lymphoid organs, including the photoconverted inguinal lymph node (Fig. 2*D*). This suggests that the rapid cellular migration reaches an equilibrium within 3 days and that normal lymph node cells with photoconverted Kaede are distributed to all lymphoid organs with no apparent loss of photoconverted Kaede signals 7 days after photoconversion (Fig. S2).

The accumulation of a large number of Kaede-positive cells in the ipsilateral axillary lymph node from the photoconverted inguinal lymph node prompted us to elucidate the possible connection between these two lymph nodes. Patent blue injection into the inguinal lymph node revealed rapid movement of the dye to the axillary lymph node via lymph, indicating a direct connection (Fig. 3 *A* and *B*). Furthermore, lymphocytes from the photoconverted inguinal lymph node could be detected in both T cell (Fig. 3 *C* and *D*) and B cell (Fig. 3 *E* and *F*) areas of the ipsilateral axillary lymph node as early as 6 h after photoconversion.

The migration of lymphocyte subsets was monitored with the same method 1 day after photoconversion. As shown in Fig. 4*A*, 64% of cells in the photoconverted inguinal lymph node were replaced, and a small number of photoconverted lymphocytes were found in other lymph nodes (pool of cells from peripheral lymph nodes except ipsilateral axillary lymph node). When the

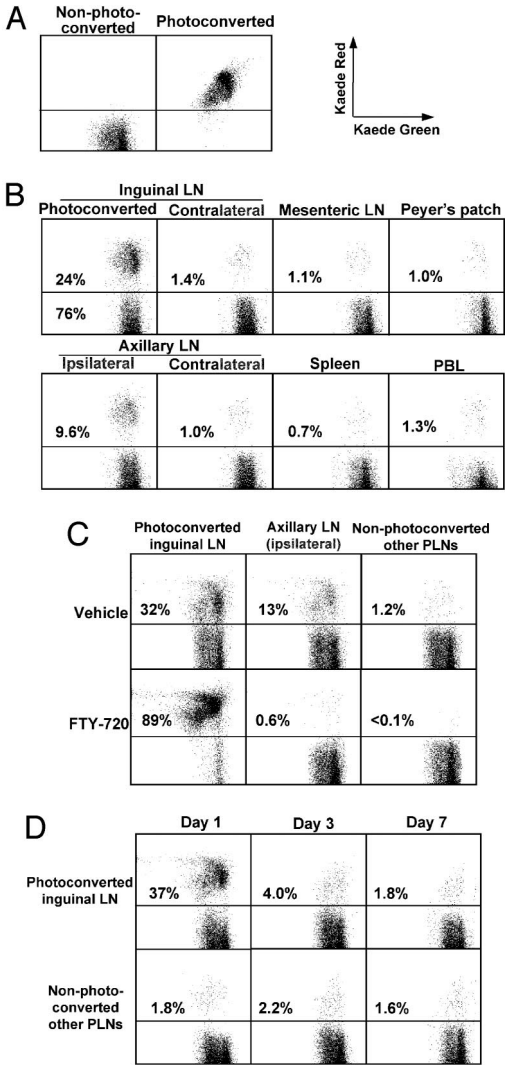


**Fig. 2.** Movement of photoconverted Kaede-positive cells in the inguinal lymph node. (*A*) Cells from nonphotoconverted and photoconverted (exposure to violet light for 5 min as described in *Materials and Methods*) inguinal lymph nodes were subjected to flow cytometry. (*B*) The inguinal lymph node was exposed in the same manner as above, and the wound was surgically closed on day 0. One day after photoconversion, cells from peripheral blood (PBL) and different lymphoid organs as indicated were subjected to flow cytometry. Three animals were independently analyzed, and the results of one representative analysis are shown. (*C*) FTY-720 (1 mg/kg) was administered i.p. to Kaede transgenic mice 4 h before photoconversion of the inguinal lymph node. Twenty-four hours after photoconversion, the photoconverted inguinal lymph node and the pool of nonphotoconverted other peripheral lymph nodes were analyzed. Control mice received vehicle only. (*D*) The inguinal lymph node was exposed to violet light, and the wound was closed as indicated in *B*. On days 1, 3, and 7 after photoconversion, the presence of photoconverted cells in the original photoconverted inguinal lymph node (LN) and nonphotoconverted other peripheral lymph nodes (PLNs) (pool of contralateral inguinal and axillary lymph nodes and bilateral popliteal, brachial, and cervical lymph nodes) was analyzed. Three animals were independently analyzed for each group with similar results, and the results of one representative analysis are shown.

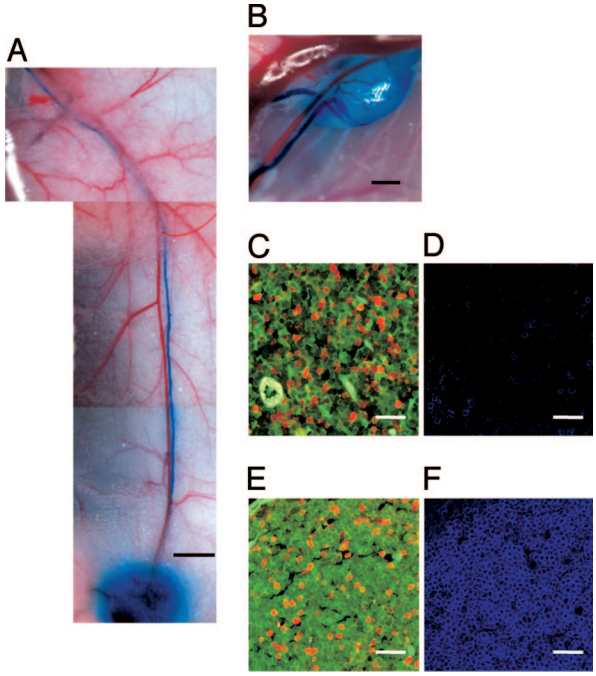


**Fig. 3.** Anatomical connection between inguinal and axillary lymph nodes via lymph. The inguinal lymph node was visualized in the same manner as that shown in Fig. 2. Patent blue was injected into the inguinal lymph node to visualize lymph. Five minutes after injection lymph from inguinal to axillary lymph node was observed as a blue line (*A*). (Scale bar: 5 mm.) (*B*) After removing connective tissue to visualize the ipsilateral axillary lymph node and lymph, lymph from the inguinal lymph node (blue) connected to the ipsilateral axillary lymph node was observed. (Scale bar: 0.5 mm.) (*C–F*) Immunofluorescence microscopy of frozen sections of the axillary lymph node 6 h after photoconversion of the inguinal lymph node. (*C* and *E*) Frozen sections (merging green and red signals). (*D* and *F*) Serial frozen sections stained with APC-conjugated anti-B220 mAbs (blue). (Scale bars: 30 $\mu$m.)

replacement rates of the lymphocyte subsets were determined (Fig. 4*A*), it became clear that each lymphocyte subset had a distinct replacement rate. $CD4^+$ T cells had the highest replacement rate (74%), followed by $CD8^+$ T cells (67%), whereas B cells showed the lowest replacement rate (49%) in the photoconverted inguinal lymph node. In other lymph nodes, the percentage of photoconverted Kaede-positive $CD4^+$ T cells was higher than that of $CD8^+$ T and B cells, reflecting the different replacement rates in the inguinal lymph node.

The time course of replacement and immigration of inguinal lymph node cells demonstrated that, as early as 6 h after photoconversion, ≈50% of $CD4^+$ T cells were already replaced (Fig. 4*B*). $CD8^+$ T cell replacement was slightly slower than that of $CD4^+$ T cells, and B cell replacement was significantly delayed compared with that of T cells. More interestingly, when the immigration of lymphocytes from the inguinal lymph node to the connecting axillary lymph node was analyzed in the same time course, $CD4^+$ T cell migration peaked at 6 h and decreased thereafter. This suggests that the migration of nonphotoconverted $CD4^+$ T cells from the photoconverted inguinal lymph node became dominant and the photoconverted $CD4^+$ T cells started to leave the axillary lymph node. Similar kinetics with a peak time of 12 h was observed for $CD8^+$ T cells, confirming the different replacement kinetics of these two subsets. B cell migration to the axillary lymph node was significantly slower than T cell migration and did not peak at 24 h after photoconversion. It should be noted that, in other lymph nodes that received photoconverted cells from bloodstream, the migration of cells from the inguinal lymph node was delayed and was still increasing at 24 h after photoconversion.

**Migration of Dendritic Cells *in Vivo* in the Steady State.** Dendritic cell migration in the inguinal lymph node was also monitored with the same method. However, because of the high Kaede expression in dendritic cells that spilled to photoconverted Kaede

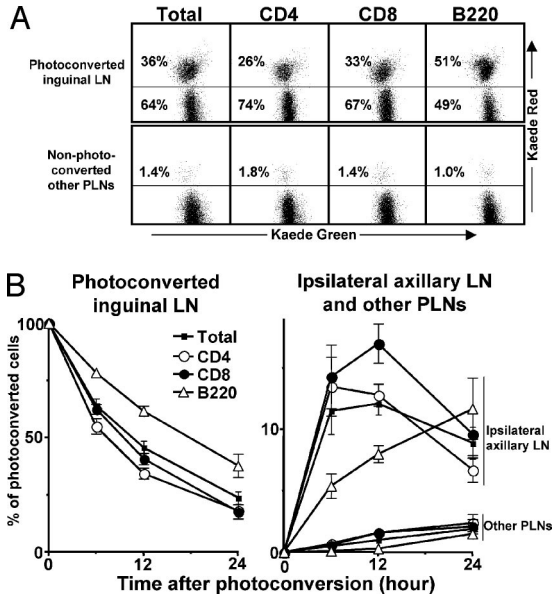


**Fig. 4.** Movement of lymphocyte subsets in the inguinal lymph node. (*A*) The inguinal lymph node was exposed to violet light in the same manner as that shown in Fig. 2*A*. One day after photoconversion, cells from photoconverted inguinal lymph node (photoconverted inguinal LN) and the pool of nonphotoconverted other peripheral lymph nodes (nonphotoconverted other PLNs) were stained with indicated Abs (APC-conjugated anti-CD4, anti-CD8, or anti-B220 mAb) and subjected to flow cytometry. (*B*) The inguinal lymph node was exposed to violet light in the same manner as that shown in Fig. 2*A*. At indicated time points after photoconversion, cells from photoconverted inguinal lymph node, ipsilateral axillary lymph node, and the pool of nonphotoconverted other peripheral lymph nodes were stained according to the method described in *A*. More than four mice were independently analyzed for each group (mean ± SE).

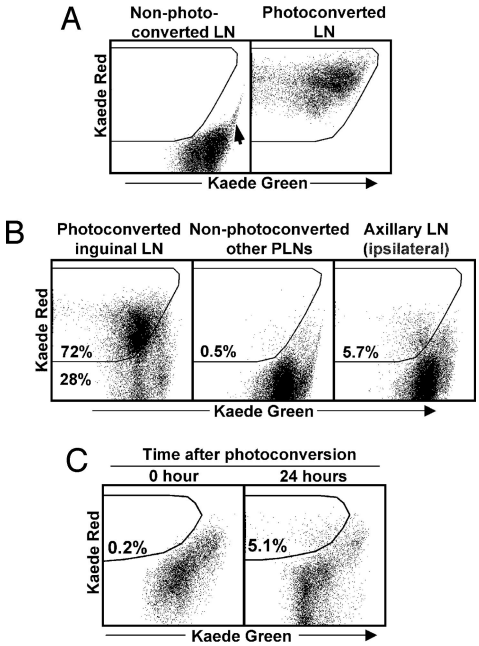


**Fig. 5.** Migration of dendritic cells in the steady state. (*A*) CD11c$^+$ cells from photoconverted inguinal lymph node (immediately after exposure to violet light for 5 min) and nonphotoconverted inguinal lymph node were gated and analyzed for Kaede signals. (*B*) The inguinal lymph node was photoconverted with the same methods as those in Fig. 2*A*. Twenty-four hours later, CD11c$^+$ cells from photoconverted inguinal lymph node, ipsilateral axillary lymph node, and the pool of nonphotoconverted other peripheral lymph nodes were subjected to flow cytometry. Four animals were independently analyzed with similar results, and the results of one representative analysis are shown. (*C*) Dorsal skin of mice was exposed to violet light. Immediately (0 h) or 24 h after photoconversion, dendritic cells in draining brachial lymph node were gated by means of anti-CD11c mAb staining and analyzed for the presence of photoconverted Kaede-positive skin-derived cells. Four animals were independently analyzed for each group with similar results, and the results of one representative analysis are shown.

signal, a different gate setting was necessary for the analysis (see high Kaede green cells showing Kaede red signals in nonphotoconverted lymph node cells indicated by an arrow in Fig. 5*A*). In the steady state, dendritic cells in the inguinal lymph node were replaced at a much lower rate than lymphocytes (28% for dendritic cells in Fig. 5*B* and 76% for lymphocytes in Fig. 4*B*). Moreover, very few migrating dendritic cells were found in the lymph node except the connecting ipsilateral axillary lymph node. A small but detectable number of CD11c$^+$ cells from the photoconverted inguinal lymph nodes were also found in other lymph nodes (Fig. 5*B*), spleen, bone marrow, liver, and lung, but not in thymus (Table S1). Taken together, it is clear that cells in the immune system are rapidly moving from one lymphoid organ to another, but each cell type has its own replacement rate in the peripheral lymph node.

The movement of dendritic cells from nonlymphoid organs to the draining lymph node was analyzed by examining the photoconversion of skin resident cells (Fig. 5*C*). Exposure of dorsal skin to violet light did not generate photoconverted Kaede-positive cells in the draining brachial lymph node. However, 24 h after photoconversion of the skin, 5.1% of dendritic cells in the draining brachial lymph node were positive for photoconverted Kaede. These results demonstrate that Kaede transgenic mice can be used to monitor the migration of cells from nonlymphoid tissue to lymphoid organs under noninflammatory conditions.

## Discussion

Cellular movement in the immune system *in vivo* has been studied by (*i*) marking cells *ex vivo* with various methods, (*ii*) injecting the cells into animals, and (*iii*) detecting injected cells at different anatomical sites at different times after injection (9–13). Using a combination of these conventional methods and cells from genetically modified mice as well as new methods to label cells *ex vivo*, many studies have demonstrated the homing pattern of cells in different anatomical sites and molecules critical for such tissue-specific homing. However, the results of those studies are limited to the general homing pattern of cells with no information of cellular emigration from tissue.

We have used a transgenic mouse line carrying the photoconvertible fluorescence protein Kaede to establish a method to monitor cellular movement *in vivo* with minimum manipulation. With this mouse line it is possible to mark cells *in vivo* with violet light, which has no effect on the function of T and B cells *in vitro* (Fig. 1*D*). It should be noted that use of violet light (436 nm) rather than harmful UVA (320–400 nm) and UVB (290–320 nm) allowed us to photoconvert Kaede in the cells with no detectable damage. Once photoconverted, the signal in CD4$^+$, CD8$^+$, and B220$^+$ populations remained stable in all cell types and did not show any significant decrease 7 days after photoconversion (Fig. S2). However, the intensity of the photoconverted Kaede signal declined as cells proliferated (Fig. 1*A* and Fig. S1).

In this study, the use of Kaede transgenic mice to monitor cellular movement *in vivo* required a surgical procedure. This procedure, however, induced no obvious inflammatory response in the photoconverted lymph nodes, as proven by the lack of any differences in cell number and subset distribution between light-exposed and nonmanipulated contralateral inguinal lymph

nodes at 24 h after exposure (Fig. S4). With this method, we demonstrated that ≈70% of lymph node cells were replaced within 24 h and that the cells migrated to other lymph nodes. Three days after photoconversion, the percentage of cells with photoconverted Kaede fell to the level found in other lymph nodes, indicating that cells from the inguinal lymph node were distributed to the whole body and reached equilibrium within 3 days. This analysis revealed the direct connection of the inguinal lymph node to the ipsilateral axillary lymph node and the migration of a large number of cells from the inguinal lymph node to the axillary lymph node via lymph. However, at this point it is not clear whether this is the major emigration route for all cells in the inguinal lymph node or not. The relationship between emigration of cells from the lymph node via lymph vessel and blood vessel needs further study.

Cell-type-specific migration *in vivo* has been analyzed by intravenously injecting cells and analyzing cell populations from afferent and/or efferent lymph with chronic lymph drainage. Studies have shown that $CD4^+$ T cells transited lymph node faster than $CD8^+$ T cells and that B cell transition was slower than T cell transition in sheep (24–26) and rats (27, 28). However, measurements were carried out for the movement of cells into and from whole lymphoid organs, and there is no direct measurement of cell-type-specific movement of cells from the lymph node to other organs, including other lymph nodes *in vivo*. Our analysis, in agreement with previous studies, showed the very rapid replacement of $CD4^+$ T cells in the inguinal lymph node (50% replacement in 6 h). The replacement of $CD8^+$ T cells was slightly slower than that of $CD4^+$ T cells, and B cell replacement was significantly slower than T cell replacement. Although there are some variations in the replacement rate in different mice, this order of $CD4^+$ T → $CD8^+$ T → B cells is consistent.

Analysis of the axillary lymph node demonstrates that the direct migration of photoconverted $CD4^+$ T cells from the inguinal lymph node peaked at 6 h and the percentage of photoconverted $CD4^+$ T cells decreased. This decrease is likely due to the secondary migration of nonphotoconverted $CD4^+$ T cells from the inguinal lymph node. The migration of photoconverted $CD8^+$ T cells and B cells to the axillary lymph node also reflects the different replacement rates of cells in the inguinal lymph node. From these results, it is clear that each cell type has its own replacement and emigration rates in the lymph node. At present we do not know how this cell-type-specific migration/replacement is regulated, and our limited analysis of chemokine receptors and integrin expression did not yield any meaningful results (data not shown).

Dendritic cell replacement is slower than T and B cell replacements in normal mice; however, 30% of them were replaced daily in the inguinal lymph node. In agreement with previous reports (29, 30), we also found a small number of dendritic cells migrating to other lymph nodes, bone marrow, spleen, liver, and lung from the inguinal lymph node. In contrast to Bonasio *et al.* (30), we did not detect any migration of dendritic cells to thymus (Table S1). The difference between the migrating capacity of dendritic cells in our study and that in previous studies may be due to differences in conditions and/or cell type, e.g., *in situ* labeling used in this study vs. *ex vivo* labeling of different types of dendritic cells for injection and parabiosis used in previous studies (29, 30).

Exposure of skin to violet light allowed us to analyze the movement of tissue (skin) dendritic cells to the draining lymph node. A similar photoconversion method was also applied to gut and other organs, and movement of cells into and out of these organs is currently being studied. In the draining brachial lymph node, ≈5% of dendritic cells migrated from skin daily in the steady state (Fig. 5*C*). Thus, although there is a possibility that nonphotoconverted dendritic cells also migrate from skin to the draining lymph node, we can conclude that a large number of dendritic cells migrate into the lymph nodes from skin in nonimmunized conditions. Taken together Kaede transgenic mice would allow us to study the function and movement of dendritic cells in different anatomical sites before, during, and after inflammatory/antigen stimulation. In fact, we found that, under inflammatory conditions, similar percentages of skin-derived dendritic cells could be detected in draining lymph nodes by photoconversion of skin in Kaede transgenic mice and traditional FITC painting of normal mice (Fig. S5).

In this study we established a method to monitor cellular movement *in vivo* using the Kaede transgenic mouse line. This method requires very little external manipulation and allows us to monitor both cellular immigration to and emigration from lymphoid and nonlymphoid organs. We expect that the Kaede transgenic mouse line would be a useful tool to monitor precise cellular movement at different stages of immune response. It should also enable us to study cellular movement during infection with pathogens and the development of autoimmune disease. Such information, in combination with the use of the two-photon laser intravital microscope to study cellular interactions, would provide new insights to the initiation and maintenance of immune responses *in vivo*.

## Materials and Methods

**Mice and Cell Line.** Kaede transgenic mice generated from $BDF_1$ (our unpublished observations) and back-crossed to C57BL/6 more than six times and used. C57BL/6 mice were obtained from CREA Japan. The mice were maintained under specific pathogen-free conditions, and experiments were carried out in compliance with institutional guidelines. The human cervical carcinoma cell line HeLa (American Type Culture Collection) was transfected with CMV-Kaede-cDNA plasmid by FuGENE6 (Roche Diagnostics).

**Photoconversion.** Kaede photoconversion in HeLa cells was done with a Leica fluorescence microscope by 10-s exposure through a UV light bandpass filter (360/40 nm, 100-W mercury lamp, ×20 objective). For photoconversion of neonatal mice, mice were exposed to violet light through a bandpass filter (400/30 nm) with Spot UV curing equipment (SP250; Usio). For photoconversion of inguinal lymph node, Kaede transgenic mice were anesthetized and abdominal skin was cut at the midline to visualize the inguinal lymph node. After the surrounding tissue was covered with aluminum foil, the lymph node was exposed to violet light (95 mW/cm$^2$ with a 436-nm bandpass filter with Spot UV curing equipment: SP500; Usio) through a hole in the foil with continuous instillation of warmed PBS. Then the wound was closed with a suture. For photoconversion of skin, mice were anesthetized and shaved, and dorsal skin ≈20 mm in diameter was exposed to violet light. In some experiments, single-cell suspensions of spleen cells from Kaede transgenic mice were exposed to violet light.

**Flow Cytometry Analysis.** Cells were washed with staining buffer consisting of 2% FCS and 0.02% sodium azide in Dulbecco's PBS and stained with allophycocyanin (APC)-conjugated anti-mouse CD4, anti-mouse CD8, or anti-mouse B220 (BD Pharmingen) for 15 min on ice. In some experiments, cells were treated with 2.4G2 hybridoma culture supernatant to block Fc binding and stained with APC-conjugated anti-mouse CD11c (BD Pharmingen). 7-Amino-actinomycin D (BD Pharmingen) was added to the sample to gate out dead cells. Stained samples were analyzed on a FACSCalibur (BD Pharmingen). Fluorescence signals of nonphotoconverted and photoconverted Kaede were detected with FL1 and FL2 channels, respectively. Flow cytometry data were analyzed with Flowjo software (Tree Star).

**Proliferation Assay.** An indicated number of spleen cells were cultured with Con A (5 μg/ml) or LPS (10 μg/ml) in a flat-bottomed 96-well plate for 48 h and pulsed with 37 kBq of [$^3$H]thymidine for the last 8 h. Cell proliferation was determined by [$^3$H]thymidine incorporation.

**Visualization of Lymph.** Mice were anesthetized, and lymph visualization was carried out by injecting Patent Blue (Wako Pure Chemical, 2% in PBS) into the inguinal lymph node.

**Immunofluorescence.** Lymph node was removed, fixed with 4% paraformaldehyde in PBS, and exchanged with 16% sucrose in PBS. Frozen sections were

stained with APC-conjugated anti-B220 mAbs for visualizing B cells. The samples were observed with a confocal microscope (Leica, SP5) and illuminated with a laser with 488 nm for green Kaede, 561 nm for photoconverted Kaede, and 633 nm for APC, respectively; these emission signals were obtained by setting the wavelength range at 500–550 nm, 570–620 nm, and 645–700 nm, respectively.


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